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Article (Published Version)

Journal of Sugarcane Research (2013) 3 (2) : 141-144

RESEARCH ARTICLE

PROSPECTING FOR SCARABID SPECIFIC *BACILLUS THURINGIENSIS* CRYSTAL TOXIN *CRY8* GENE IN SUGARCANE ECOSYSTEM OF TAMIL NADU, INDIA

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Abstract

In the present study, we report the occurrence of *cry8* positive isolates of *Bacillus thuringiensis* (*Bt*) in selected white grub, *Holotrichia serrata* F. (Coleoptera: Scarabaeidae), endemic soils of sugarcane ecosystem and other places in Tamil Nadu. Out of the 66 soil samples collected and screened for white grub specific *Bt*, 74 isolates of the bacterium, all containing only spherical crystal toxin, were identified. PCR screening of these isolates with *cry8* gene universal primer revealed six isolates to be positive. Further, the amplicon of a 370 bp band, amplified with another set of degenerate primer designed based on the conserved sequence of *cry8* genes, was sequenced from four isolates. Multiple sequence alignment revealed the gene sequences to be the same for all the isolates. The present report of the availability of *cry8* positive *Bt* isolates opens the avenue for controlling white grubs through transgenic research.

Key words: Sugarcane, white grub, *Holotrichia serrata*, Scarabaeidae, *Bacillus thuringiensis*, *cry8* gene

Introduction

Melolonthin scarabs occur across the Indian subcontinent and throughout southeast and east Asia (Ward et al. 2002). The chafer beetle *Holotrichia serrata* F. (Coleoptera: Scarabaeidae) is one such scarab whose larval stage is a serious pest of coconut (*Cocos nucifera* L.), sugarcane (*Saccharum ofûcinarum* L.), groundnut (*Arachis hypogaea* L.) and vegetables in parts of Western and peninsular India (Subaharan et al., 2013). The grubs of *H. serrata* damage sugarcane crop by feeding on the roots ultimately leading to drying of the plant, often causing 80-100% crop loss (David and Ananthanarayana 1986). The subterranean habit makes this pest intractable for control and hence the available control measures are less than satisfactory. Among the various control options, biocontrol agents such as the entomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Srikanth et al. 2009) and entomopathogenic nematode *Heterorhabditis indicus* (Sankaranarayanan et al. 2006) hold some promise although slow speed of kill by the fungus and lack of economically viable mass multiplication techniques of the nematode limit their use. The use of the soil bacterium *Bacillus thuringiensis* (*Bt*) against this serious pest has not been contemplated

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in our country as was evident from the lack of reports. However, recently *Bt* that have specific mode of action against other *Holotrichia* species have been isolated in China. The *Bt* isolated from China was found to harbour Cry8-type toxin with known specific action against scarab pests (Yu et al. 2006; Shu et al. 2009a & 2009b). In the present study, *Bt* strains were isolated from the soils of sugarcane ecosystem and screened for the presence of *cry*8 genes, which encode insecticidal proteins that are toxic to scarab insects like *H. serrata*.

Materials and methods

Soil collection

Soil samples for isolation of *Bt* were collected from white grub endemic areas of Tamil Nadu, namely Anaikatty Hills, Coimbatore district, and Sakthi Sugars Ltd and Bannari Amman Sugars Ltd, Erode district (Table 1). Fifty grams of soil samples were collected by scraping off surface soil with a spatula upto a depth of 30 cm. Soil samples were collected in polyethylene bags, transferred to the lab and stored at room temperature.

Bt isolation

Bt isolation from soils was carried out with a slight modification to the method followed by Travers et al. (1987). Ten grams of soil sample was suspended in 100 ml of 0.85% NaCl solution. The samples were heated at 80°C for 15 min in water bath. One ml of the heat treated sample was inoculated in 50 ml of Luria Bertani (LB) broth and incubated overnight at 30°C with an orbital shaking of 250 rpm. From this broth, serial dilution (10^{-1} to 10^{-6}) was performed and each dilution was plated in Travers (T3) medium. The plates were then incubated for 48h at 30°C. After incubation, *Bt* like colonies were selected and streaked on T3 and incubated for 72h at 30°C. The single colonies were observed under phase contrast microscopy for identification of *Bt*. The isolates showing the presence of crystalline inclusions were selected as *Bt* and streaked on T3 agar medium for single colony purification. Broth culture (pH adjusted to 6.9) was made from the isolated single colonies of crystal positive *Bt* isolates. Glycerol stocks of *Bt* isolates were prepared by using equal amounts of 30% glycerol and 72 h old T3 broth culture and stored at -20°C for further studies.

PCR screening of *cry8* gene

Polymerase chain reaction (PCR) was used to identify *cry8* gene type from our isolates. *Bt* reference *cry8* strain (Buibui) and our isolates from white grub endemic soils were streaked on Luria Agar (LA) plate and grown overnight at 30°C. Two swipes of the overnight grown cultures were suspended in 200 µl of sterile distilled water and the isolates were heated by placing them in boiling water for 10 min. The lysed cells were allowed to settle for 8-10 min at room temperature and the supernatant was taken as DNA template for PCR reaction. Universal primers (forward and reverse) of *cry*8 were used to amplify a specific fragment as described by Bravo et al. (1998). All PCR reactions were carried out in 50 µl reaction volumes.

Table 1. Details of locations, sugarcane soil samples and *Bacillus thuringiensis* isolated in Tamil Nadu

S. No.	Location	No. of soil samples	No. of colonies selected	No. of *Bt*s isolated
1	Anaikatty, Coimbatore	6	100	4
2	Sakthi Sugar Mills Ltd, Tamil Nadu	31	682	34
3	Bannari Amman Sugar Mills Ltd, TamilNadu	29	427	36

Twenty µl of template DNA was mixed with reaction buffer containing 1.25 µl of 2.5mM deoxynucleoside triphosphate mix, 0.5 µl of 10µM (direct and reverse) primers, 5 µl Taq buffer (10 X), and 1 U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Biorad S1000). The PCR conditions for screening the isolates were as follows: single denaturation step of 5 min at 94°C, a step cycle program set for 30 cycles (with a cycle consisting of denaturation at 94°C for 1 min, annealing at 50°C for 30 seconds and extension at 72°C for 1 min), and an extra step of extension at 72°C for 7 min after completion of all the cycles. All PCR reactions were performed with the *cry8* reference strain *B. thuringiensis* serovar *japonensis* Buibui. Following the amplification, electrophoresis of each PCR sample was done on 1 % agarose-ethidium bromide gel.

Results and discussion

Out of the 66 soil samples collected from white grub endemic areas, 74 isolates were identified as *Bt* (Table 1) by phase contrast microscopy. *Bt*s are generally identified by their crystal toxins which are easily visible under phase contrast microscope. In our study all the *Bt* isolates were found to contain only spherical crystal toxin (Fig.1).

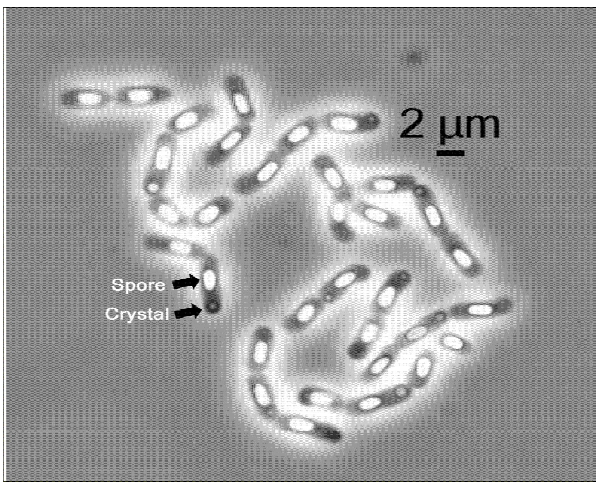

Fig. 1. A *Bt* isolate from sugarcane soil showing spherical crystal toxin under phase contrast microscope (1000x magnification)

PCR screening of the 74 isolates of *Bt* identified in this study with *cry8* gene universal primer revealed that six isolates, namely *Bt* 56, 57, 58, 59, 61 and 62 were positive (Fig 2). Since all the *cry8* positive isolates were isolated from the same soil sample, we amplified a 370 bp band with another set of degenerate primer designed based on the conserved sequence of *cry8* genes. This PCR amplicon from *Bt* 56, 58, 61 and 62 was sequenced and multiple sequence alignment carried out by CLUSTAL W revealed the gene sequences to be the same for all the isolates indicating they all could be the same isolate. The availability of *cry8* positive *Bt* isolates opens the avenue for controlling white grubs through transgenic research. However, further studies on the toxicity of these isolates to white grubs, and cloning and sequencing of the entire gene are required to know whether the isolate identified in the present study contains crystal toxin gene that matches with *cry8* holotype genes described earlier or it expresses a novel *cry8* gene.

The number of *Bt* isolates obtained from the three locations (Table 1) appeared to be positively related to the number of soil samples collected. Thus, more extensive sampling of soils is likely to increase the probability of identifying new *Bt* isolates, including those with *cry8* gene. The occurrence of *Bt* isolates

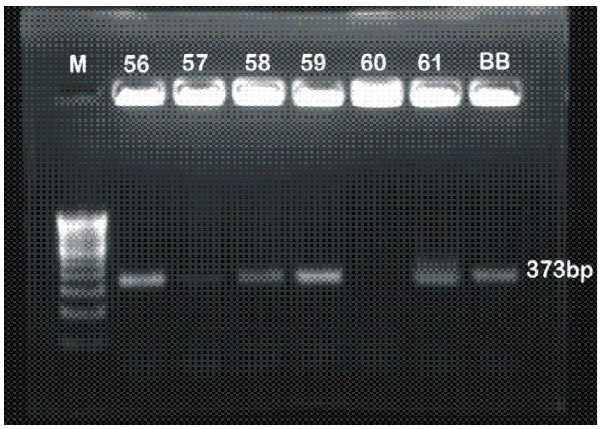

Fig. 2. PCR screening of *Bt* isolates with universal primers. M: marker; 56-61: *Bt* isolates; BB: Buibui strain of *B. thuringiensis* serovar *japonensis*

in soils from location 1 (Anaikatty), which is a forest area and not a true sugarcane crop ecosystem, indicated that the search for *Bt* isolates need not be restricted to sugarcane crop area alone but can be extended to other crop systems as well as natural systems, which could be a rich repository of isolates of the bacterium.

Acknowledgements

The authors thank Dr N. Vijayan Nair, Director, Sugarcane Breeding Institute, Coimbatore, for providing facilities to carry out this work. The authors also thank Dr M. Mohan, Senior Scientist, Division of Molecular Entomology, National Bureau of Agriculturally Important Insects, Bangalore, for providing the *cry8* gene reference isolate. The authors also thank Neil Crickmore, Senior Lecturer in Molecular Genetics, Department of Biochemistry, School of Life Sciences, University of Sussex, Brighton, United Kingdom, for the technical guidance rendered in sequencing and multiple sequence alignment. The role of National Agricultural Innovation Project (NAIP) International training program under Human Resource Development Programme is also gratefully acknowledged.

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